Salvage of Contaminated Osteochondral Allografts

The Effects of Chlorhexidine on Human Articular Chondrocyte Viability

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Background: Because chondrocyte viability is imperative for successful osteochondral allograft transplantation, sterilization techniques must provide antimicrobial effects with minimal cartilage toxicity. Chlorhexidine gluconate (CHG) is an effective disinfectant; however, its use with human articular cartilage requires further investigation.

Purpose: To determine the maximal chlorhexidine concentration that does not affect chondrocyte viability in allografts and to determine whether this concentration effectively sterilizes contaminated osteoarticular grafts.

Study Design: Controlled laboratory study.

Methods: Osteochondral plugs were subjected to pulse lavage with 1-L solutions of 0.002%, 0.01%, 0.05%, and 0.25% CHG and cultured for 0, 1, 2, and 7 days in media of 10% fetal bovine serum and antibiotics. Chondrocyte viability was determined via LIVE/DEAD Viability Assay. Plugs were contaminated with *Staphylococcus aureus* and randomized to 4 treatment groups. One group was not contaminated; the 3 others were contaminated and received no treatment, saline pulse lavage, or saline pulse lavage with 0.002% CHG. Serial dilutions were plated and colony-forming units assessed.

Results: The control group and the 0.002% CHG group showed similar cell viability, ranging from 67% ± 4% to 81% ± 22% (mean ± SD) at all time points. In the 0.01% CHG group, cell viability was reduced in comparison with control by 2-fold at day 2 and remained until day 7 ($P < .01$). The 0.05% and 0.25% CHG groups showed a 2-fold reduction in cell viability at day 1 ($P < .01$). At day 7, cell viability was reduced to 15% ± 18% (4-fold decrease) for the 0.05% CHG group and 10% ± 19% (6-fold decrease) for the 0.25% CHG group ($P < .01$). Contaminated grafts treated with 0.002% CHG demonstrated no colony-forming units.

Conclusion: Pulse lavage with 0.002% CHG does not cause significant cell death within 7 days after exposure, while CHG at concentrations >0.002% significantly decreases chondrocyte viability within 1 to 2 days after exposure and should therefore not be used for disinfection of osteochondral allograft. Pulse lavage does not affect chondrocyte viability but cannot be used in isolation to sterilize contaminated fragments. Overall, 0.002% CHG was shown to effectively decontaminate osteoarticular fragments.

Clinical Relevance: This study offers a scientific protocol for sterilizing osteochondral fragments that does not adversely affect cartilage viability.

Keywords: osteochondral graft; contamination; dropped; decontamination

Cartilage restoration offers treatment for localized chondral and osteochondral defects in patients who are too young for joint replacement. Such articular cartilage defects can be treated with surgical implantation of fresh osteochondral allograft tissue containing viable chondrocytes, a principal determinant for allograft selection.[14] Key steps in the procedure include graft harvest and procurement, followed by allograft preparation and implantation into the recipient defect site. Accidental graft contamination, such as mishandling in the operating room, remains a concern. Up to 25% of sports medicine surgeons experience at least 1 contamination event in their practice.[9]

Staphylococcus aureus has been shown to be the most common orthopaedic surgical site infection.[1,11] Furthermore, it has been identified on skin flora as well as on

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the operating room floor.[1] The deleterious effects of methicillin-resistant *S aureus* (MRSA) on postoperative orthopaedic patients are significant from both a clinical and a financial perspective.[6,8,13,17] Efforts to minimize MRSA contamination for orthopaedic surgery have been shown to be effective in preventing surgical site infections.[8]

Multiple methods for decontaminating orthopaedic implants have been studied. Chlorhexidine has been shown to be a valuable agent for bacterial decontamination.[12,15] Previous work with anterior cruciate ligament allograft decontamination procedures has demonstrated that a chlorhexidine solution is an efficacious antimicrobial agent when compared with povidone-iodine and triple-antibiotic solutions.[4,10] Povidone-iodine has been shown to decontaminate with minimal chondrocyte toxicity in some studies; however, others have contrarily demonstrated that wet povidone-iodine solutions did not effectively decontaminate bone fragments, even after 10 minutes of irrigation.[2,4,18] Overall, a multitude of studies have collectively concluded that chlorhexidine is more effective than povidone-iodine.

When further detailing the use of chlorhexidine in irrigation, it is important to consider that its use, in some cases, has been associated with the development of severe chondrolysis in anterior cruciate ligament graft transplantation.[5,7] Bruce et al[4] further described this by demonstrating that 1-minute exposure of bone fragments to 4% chlorhexidine resulted in almost complete chondrocyte death. Recent studies using 0.05% chlorhexidine, however, have shown that 1-minute jet lavage of articular cartilage does not have a significant effect on chondrocyte metabolism as measured by radiolabeled sulfur uptake.[8,17] This method and determined 0.05% chlorhexidine concentration have proven effective in removing or killing up to 99.8% of contaminating bacteria.[3,16]

Although other studies support the use of 0.05% chlorhexidine jet lavage for osteochondral allograft decontamination procedures, they are limited in that the analysis of chondrocyte metabolism was not continued beyond 24 hours. Furthermore, the articular cartilage tested in these studies was obtained immediately after orthopaedic surgery, while cartilage transplantation procedures utilize prolonged (14-28 days) fresh cold-preserved cadaveric donor tissue.[19,20] In addition, to our knowledge, the effects of different concentrations of chlorhexidine jet lavage on articular cartilage have not been investigated.

The purpose of this study was to identify the maximal chlorhexidine concentration that would not affect chondrocyte viability in fresh human osteochondral allografts and to subsequently determine the effectiveness of decontamination. We hypothesized that chlorhexidine pulse lavage with concentrations of 0.05% or less will not significantly affect chondrocyte viability but will effectively sterilize contaminated allograft fragments.

MATERIALS AND METHODS

This investigation represents a 2-phase study in which the first phase identifies a concentration of chlorhexidine gluconate (CHG) that does not adversely affect chondrocyte viability. The goal of the second phase is to determine whether this concentration of CHG effectively sterilizes contaminated osteochondral fragments.

Phase 1: Determination of Cytotoxic Concentration of CHG

Experimental Design. Five human femoral hemicondyles, refrigerated at 4°C for 14 to 28 days, were obtained from AlloSource (Centennial, Colorado, USA) (Figure 1, A-F). A total of 48 six-millimeter osteochondral plugs were harvested with the osteochondral autograft transfer system technique (Arthrex Inc, Naples, Florida, USA) and randomized to the following treatment groups: untreated control, saline pulse lavage, 0.002% chlorhexidine pulse lavage, 0.01% chlorhexidine pulse lavage, 0.05% chlorhexidine pulse lavage, and 0.25% chlorhexidine pulse lavage. These concentrations were chosen because each represents a 5-fold reduction in chlorhexidine concentration ($0.25 > 0.05 > 0.01 > 0.002$), with base concentration being 0.05. Cell viability of cartilage from osteochondral plugs was analyzed at selected time points of 0, 1, 2, and 7 days after treatment with LIVE/DEAD Cell Viability Assay (Molecular Probes Inc, Eugene, Oregon, USA). There were 8 plugs per group with 2 plugs per time point.

Osteochondral Plug Harvest. Hemicondyles were held in place with bone clamps, and the osteochondral autograft transfer system harvester was oriented at a 90° angle to the articular surface and advanced with a mallet to a depth of 10 mm. The harvester with the osteochondral plug was removed by axial loading the harvester and rotating 90° clockwise, then 90° counterclockwise. The core extruder was then inserted and advanced to atraumatically eject the plug (Figure 1, A-F). Plugs were then placed in culture containing 10% fetal bovine serum in standard 50/50 Dulbecco's Modified Eagle's Medium/F12 nutrient mixture and penicillin/streptomycin/fungizone/gentamicin at room temperature and treated immediately after procurement.

Chlorhexidine Pulsatile Lavage. Two controls were included: osteochondral plugs immediately cultured after harvest (untreated control) and plugs subjected to saline

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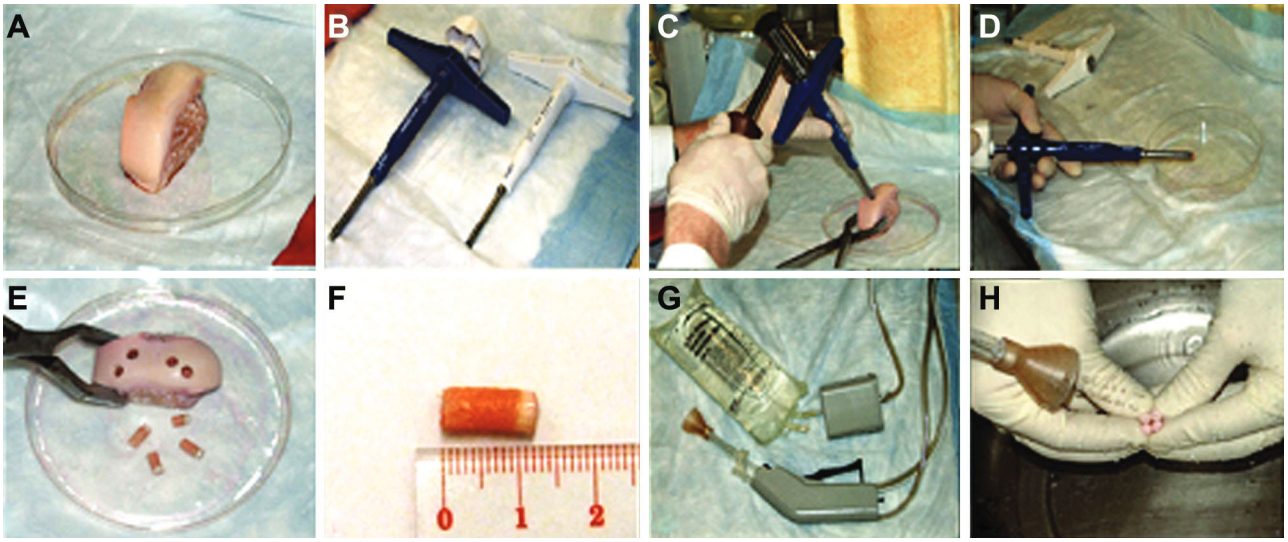

Figure 1. (A) Femoral hemicondyle, (B) osteochondral autograft transfer system harvesters, (C) graft harvest, (D) plug removal using core extruder, (E) hemicondyles with osteochondral plugs, (F) osteochondral plug, (G) pulse lavage system with saline bag, (H) pulse lavage of osteochondral plugs.

pulse lavage only before culture (saline pulse lavage). The saline pulse lavage group was washed with 3 L of saline before culture in 10% fetal bovine serum media. Pulse lavage was performed with sterile technique via a battery-powered, variable-speed Stryker InterPulse hand piece (Stryker, Kalamazoo, Michigan, USA) spiked into 1-L saline bags and operated at maximum speed, with each 1-L bag lasting approximately 1 minute (Figure 1, G and H). All surfaces were equally exposed to lavage. Osteochondral plugs from treatment groups were subjected to pulse lavage with 1-L solutions of 0.002%, 0.01%, 0.05%, or 0.25% CHG, preceded and followed by 1-L saline pulse lavage (3-L total volume). Chlorhexidine solutions were created by injecting calculated volumes of a 20% CHG solution (Xttrium Laboratories, Chicago, Illinois, USA) into 1-L bags of sterile water to achieve desired concentration. After treatment, plugs were cultured in 10% fetal bovine serum media at 37°C until selected time points (0, 1, 2, and 7 days) for analysis.

Determination of Chondrocyte Viability. At time points (0, 1, 2, and 7 days), cartilage (full thickness) was removed from subchondral bone and cell viability analyzed with LIVE/DEAD Viability Assay. This is a 2-color fluorescence cell viability assay based on the determination of live and dead cells through simultaneous use of calcein AM and ethidium bromide homodimer 1 stains to measure intracellular esterase activity and plasma membrane integrity. Live cells emit a green fluorescence after intracellular esterases convert calcein to fluorescent calcein. Ethidium bromide homodimer 1 enters cells upon the disruption of the plasma membrane integrity after cell death, staining the nuclear DNA red. Images were visualized via fluorescent microscopy with Image J software (National Institutes of Health, Bethesda, Maryland, USA) and Metamorph software (version 6.1r6; Molecular Devices, Sunnyvale, California, USA). Images were assigned random numbers, and live and dead cells were counted twice per image and averaged. Cell viability was determined as follows: number of live cells/total cells.

Phase 2: Determination of Efficacy of CHG Treatment in Sterilizing Contaminated Grafts

Twelve 6-mm osteochondral plugs were harvested from fresh hemicondyles and subjected to antibiotic cocktail treatment as detailed above. Osteochondral grafts were immersed in regular media 5-mL baths containing 1.4E5 bacteria colony-forming units (CFUs) of Newman strain of MRSA for 1 minute. Control cells were similarly exposed to sterile regular media. Osteoarticular fragments were subsequently removed and randomly subjected to 1 of 3 treatments: no wash, pulse lavage with saline, or pulse lavage with saline and 0.002% CHG. This concentration of CHG was based on data obtained from phase 1. The grafts were sterilely transferred to a 5-mL bath of regular media for 5 minutes to deposit any remaining bacteria to the media. The fragment was sterilely removed and the media incubated for 24 hours. The number of CFUs was identified after plating serial dilutions.

RESULTS

LIVE/DEAD Viability Assay was performed at 0, 1, 2, and 7 days after osteochondral plug harvest and treatment. Overlay images of live and dead chondrocytes from full-thickness specimens were compared at days 0 and 7 with qualitative evaluation of the untreated control showing similar distribution and amount of live cells (green) as well as minimal surface death (red cells) at both time points (Figure 2). The saline pulse lavage and 0.002% CHG group overlay images also showed very little

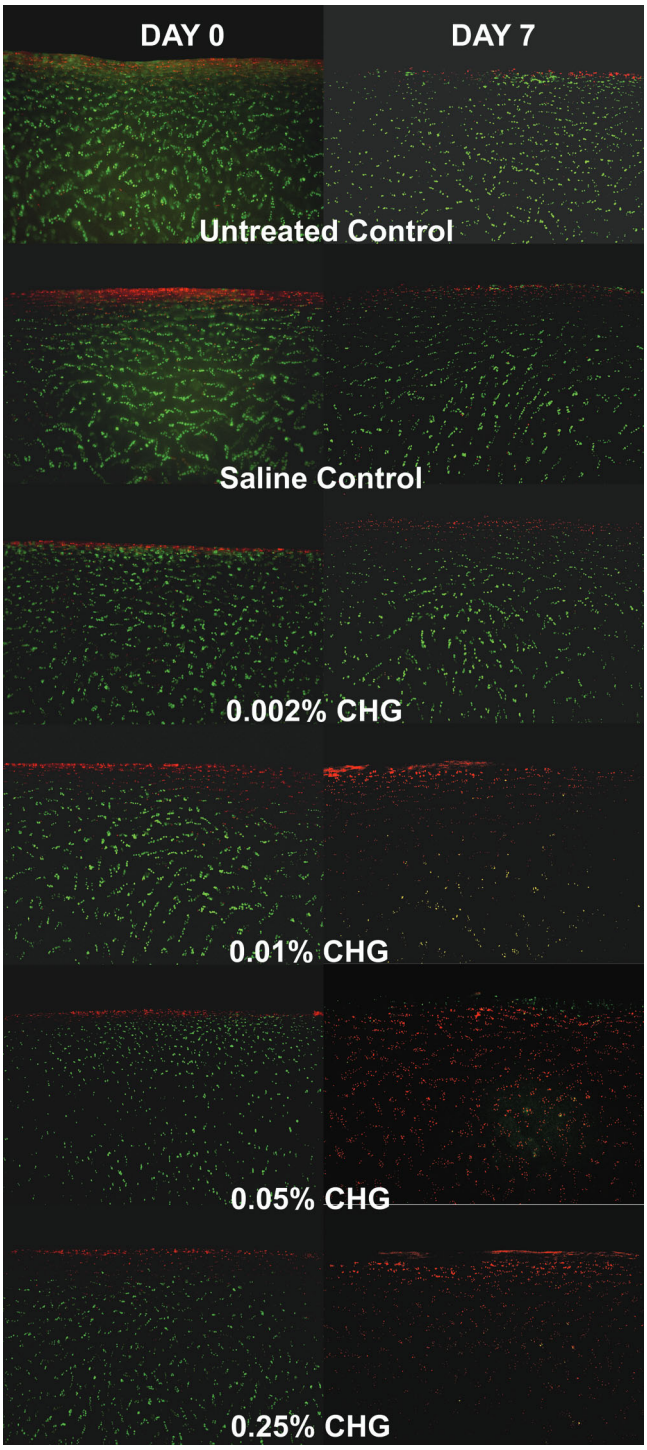

Figure 2. LIVE/DEAD overlay images for each treatment group comparing day 0 and day 7. Fluorescent green, live cells; fluorescent red, dead cells.

deviation from untreated control images with symmetrical distribution of viable cells at days 0 and 7. Similar results were observed for higher concentration of CHG at day 0, where overlay images of 0.01%, 0.05%, and 0.25% CHG groups at day 0 reported comparable cell viability to control. However at day 7, chondrocytes exposed to 0.01%, 0.05%, and 0.25% CHG showed evident decreases in cell viability as compared with day 1 images and untreated control images.

Cell viability was calculated as live cells (green fluorescence)/total cells (green + red cells) for each experimental group and time point. Untreated control, saline pulse lavage, and 0.002% CHG groups all showed similar cell viabilities, ranging from 67% ±4% to 81% ± 22% at all time points (0, 1, 2, and 7 days; no statistical significance among these groups at each time point) (Table 1, Figure 3). In the 0.01% CHG group, cell viability was reduced in comparison with the untreated control by 2-fold at day 2 and remained at this level until day 7 ($P < .01$) (Table 1, Figure 4). CHG groups 0.05% and 0.25% showed a 2-fold reduction in cell viability compared with the untreated control already at day 1 ($P < .01$). At day 7, cell viability was reduced to 15% ±18% (4-fold) for the 0.05% CHG group and 10% ±19% (6-fold) for the 0.25% CHG group ($P < .01$).

Osteochondral grafts contaminated with 1.4E5 bacterial cells that subsequently underwent no treatment or saline pulse lavage demonstrated 2.9E8 and 3.3E7 CFUs, respectively. The uncontaminated control grafts as well as contaminated grafts that were treated with 0.002% demonstrated no CFUs. LIVE/DEAD Viability Assay showed similar chondrocyte viability between 0.002% CHG and untreated control cells.

DISCUSSION

This study suggests a scientific protocol to sterilize osteochondral fragments that does not adversely affect cartilage viability. At many cartilage restoration centers, fresh cold-preserved cadaveric donor tissue is used because of the preservation of cell viability and its importance to successful graft incorporation. Osteoarticular fragments identical to those used for these procedures were utilized. Multiple studies have suggested that chlorhexidine is toxic to chondrocytes.[2,4,21] However, previous studies by Best et al,[3] using 1-minute exposures of articular cartilage to 0.05% chlorhexidine jet lavage, have shown that cartilage metabolism measured by radioactive sulfur uptake is not significantly affected. On the basis of these reports, we thought that chlorhexidine pulse lavage with concentrations of 0.05% or less would not significantly affect chondrocyte viability. However, the results from the first phase of this study confirmed otherwise.

LIVE/DEAD analysis showed that chlorhexidine pulse lavage caused significant chondrocyte death as early as day 1 after exposure to 0.05% and 0.25% CHG and at 2 days after exposure to 0.01% CHG. This onetime exposure continued to have effects on cell viability beyond the initial exposure. In our study, 1-minute 0.05% chlorhexidine pulse lavage caused a significant reduction in cell viability already by day 1 and a 4-fold reduction by day 7. This progression of cell death implies that chlorhexidine's cytotoxicity may be delayed, and early analysis of chondrocyte metabolism may not adequately reflect the detrimental effect on chondrocyte viability.

TABLE 1
Cell Viabilities (in Percentages) by LIVE/DEAD Assay for Each Experimental Group at Time Points 0, 1, 2, and 7 Days[a]

	Average Cell Viabilities of 5 Samples (2 Counts Each)			
Group	Day 0	Day 1	Day 2	Day 7
Untreated control	67 ± 11.0	68 ± 20.4	76 ± 8.5	80 ± 4.2
Saline pulse lavage	63 ± 11.2	61 ± 22.3	73 ± 12.8	81 ± 3.9
0.002% CHG	62 ± 8.4	68 ± 14.0	73 ± 7.0	68 ± 10.8
0.01% CHG	67 ± 7.0	55 ± 11.8	28 ± 17.5	28 ± 21.3
0.05% CHG	61 ± 15.3	36 ± 12.5	22 ± 13.7	15 ± 18.4
0.25% CHG	63 ± 9.2	29 ± 16.2	9 ± 7.9	10 ± 18.6

[a]CHG, chlorhexidine gluconate.

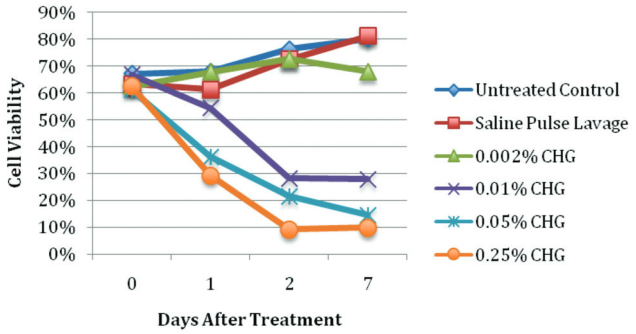

Figure 3. Phase 1: chondrocyte viabilities of groups after exposure to chlorhexidine at all time points.

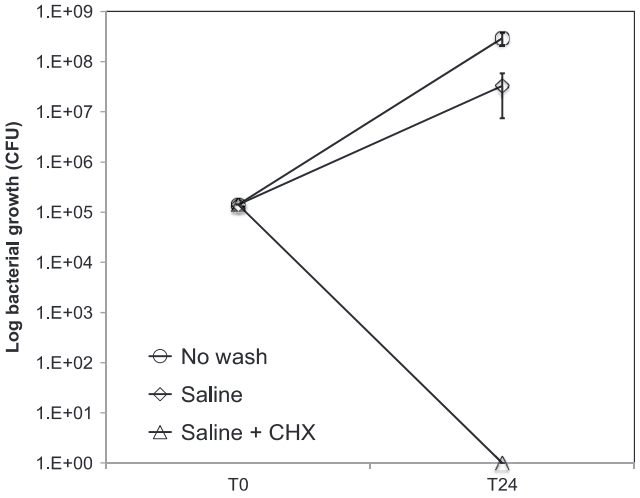

Figure 4. Phase 2: Bacterial growth assessment after treatment of contaminated osteochondral fragments with chlorhexidine. Osteochondral fragments were immersed in regular medium containing 1.0×10^5 bacteria for 1 minute (T0). Osteochondral fragments were then removed and left unwashed (no wash) or rinsed with either saline (saline) or saline + chlorhexidine at 0.002% concentration (CHX). After the rinsing step, osteochondral fragments were placed in regular medium for 5 minutes so that remaining viable bacteria could be deposited in the medium. The effect of chlorhexidine on bacterial disinfection was assessed after 24-hour growth phase (T24) in regular medium, and the data are shown as the mean ± SD (n = 3; $P < .0001$).

Untreated control, saline pulse lavage, and 0.002% CHG groups all showed similar cell viabilities at all time points analyzed, suggesting that 0.002% CHG did not compromise cell viability. The saline pulse lavage group was used as control to determine potential chondrocyte damage due to the pressurized lavage treatment and to evaluate the effects of saline on cell viability, since previous studies reported diminished chondrocyte metabolism with saline pulse lavage or mechanical scrubbing.[4] Saline pulse lavage is used in osteochondral allograft transplantation procedures to remove marrow elements from the graft before implantation and to reduce risk of potential immunogenic interactions. Our results showed that saline pulse lavage does not significantly reduce cell viability of osteochondral allograft tissue.

Furthermore, 0.002% CHG pulse lavage was found to effectively sterilize contaminated osteoarticular fragments. Chlorhexidine disrupts cell membranes leading to cell death, but to our knowledge, its antimicrobial effects at concentrations less than 0.05% have not been investigated.[7] Our study suggests that even dilute chlorhexidine effectively decontaminates nonsterile fragments. There is debate about the necessity of cell viability preservation in osteochondral allograft transplantation procedures, but this is beyond the scope of this discussion. The level of cell viability deemed adequate for successful graft incorporation remains to be determined.

There are several limitations to this study that have to be considered. In the first phase of the experiment, cartilage viability was evaluated only up to 1 week. Long-term effects of chlorhexidine exposure on chondrocytes remain to be investigated. In the second phase, the number of actual CFUs could have been underestimated, as some bacteria may remain attached to the osteochondral fragments and may cause damage at later stages or within the intra-articular environment. Second, because of specimen limitations, only MRSA was tested. Multiple other studies have confirmed that a spectrum of bacteria, including MRSA, colonize in the hospital.[11,13,15] In this experiment, MRSA was chosen because it represents one of the most common orthopaedic infections and has devastating consequences.[11] Compared

with *Staphylococcus epidermis* infection, MRSA infection is much more difficult and expensive to treat. Finally, this study does not address the in vivo risk of infection after decontamination. A randomized controlled study, however, would not be ethically feasible.

CONCLUSION

Chlorhexidine solutions of concentrations of 0.05% or above cause significant decreases in chondrocyte viability within 1 to 2 days after exposure and are not recommended for use with allograft articular cartilage; however, 0.002% CHG does not cause significant cell death within 7 days after exposure and is comparable with saline graft lavage. Isolated pulse lavage with saline does not effectively decontaminate osteoarticular fragments. Osteoarticular fragments contaminated with MRSA can effectively be decontaminated with a protocol that includes 1-L pulse lavage with 0.002% CHG, but the use of this protocol in clinics is far from being defined and thoroughly investigated.

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